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Isolation and Characterisation of Betulinic Acid, Stigmasterol and β-Sitosterol from *Calotropis procera*

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ABSTRACT

Majority of natural products are complexly structured molecules that are easily extracted, purified, and synthesized in large numbers to be used in the manufacture of pharmaceuticals and other home goods. The goal of this research project is to use proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and column chromatography to identify and analyze secondary metabolites from *Calotropis procera*. Cold maceration was used for the extraction process. The process of macerating *Calotropis procera* using hexane, ethyl acetate, and methanol in order from the non-polar hexane to the polar methanol was prior to the separation of bioactive chemicals from the plant. The extract was concentrated by employing a rotary evaporator to remove the solvent from the filtrate. Then, the concentrate was allowed to air dry and adsorbed onto silica. Isolation was done using column chromatography and thin layer chromatography. Nuclear magnetic resonance spectroscopy was used to characterize the isolated compounds. Physical and spectroscopic approaches were used for the identification of the structure. Betulinic acid and a combination of β-Sitosterol and Stigmasterol were extracted from *Calotropis procera.* By comparing the ^{1}H NMR results with those reported in literature, the characterisation was made possible. Betulinic acid and a combination of β-Sitosterol and Stigmasterol were isolated. The biological activity as well of the compounds needs to be investigated.

Keywords: Betulinic acid, *calotropis procera*, isolation, sitosterol, stigmasterol.

INTRODUCTION

Calotropis procera is primarily found in drier sections of tropical and subtropical regions, as well as semi-arid and dry inland areas [1]. It is a weed found in disturbed regions, waste places, roadsides, inland watercourses, grasslands, open forests, and pastures. It is a tiny tree or shrub that is upright, typically reaching a height of 1-4 meters, a big shrub with milky sap-filled leaves and waxy stalks. *Calotropis procera* is comparatively with big, stem-clasping, greyish-green leaves, measuring 5–20 cm in length and 4–10 cm in width, and are borne in pairs. Its flowers, which measure 20–30 mm in diameter, have five white petals with tips that are tinted purple and a centre that resembles a purplish crown. Its fruit is a big, greyish-green bladdery "pod," about 8 to 12 centimeters in length [1].

Plate 1. ***Calotropis procera* roots**

The use of medicinal plants for healing dates back as long as humanity. There is sufficient evidence from a variety of sources that links man and his search for drugs in nature to a distant past: written records, monuments that have been preserved, and even natural plant remedies [2]. The many years of fighting diseases have led man to seek out pharmaceuticals in the barks, seeds, fruit bodies, and other parts of plants. This has led to an awareness of the use of therapeutic plants. Modern science has recognised their potent effects and incorporated a variety of plant-based medications, which have been utilised for millennia by ancient societies, into contemporary pharmacotherapy [3]. Because they are aware of the developments in the theories

surrounding the use of medicinal plants and the evolution of awareness, chemists, and physicians address the issues that have emerged with the expansion of professional services in the facilitation of human life. On a Sumerian clay slab from Nagpur, the earliest known written record of the use of medicinal herbs to prepare medications dates back to 5000 years. It included twelve drug preparation formulas based on over two hundred different plants, some of which were alkaloid plants including mandrake, henbane, and poppies [4].

Natural products are chemicals or chemical combinations that are created by naturally occurring living things. The greatest sources of medications and drug leads continue to be natural items. For organic chemists, the term "natural product" refers to a wide range of substances made by living things, including lipids, proteins, carbohydrates, and nucleic acids, all of which are essential and fundamental to metabolic processes [6]. The majority of natural products are complexly structured molecules that are easily extracted, purified, and synthesized in large enough numbers to be used in the manufacture of pharmaceuticals and other practical home goods. The biosynthesis, extraction, identification, quantification, and structural elucidation of natural compounds are all included in their chemistry [7]. Both conventional and modern medicine can benefit from the use of medicinal herbs. Around 80% of people worldwide, primarily in developing nations, rely on traditional herbal therapy to manage their illnesses [8].

Since ancient times, medicinal plants have been utilized as a source of cures for various illnesses. Early people healed their sicknesses based on instinct, taste, and experience [9]. Medicinal herbs that are used to treat skin conditions include Artemisia nilagrica, Vaccinium microcarp, Senellgalia *visco*, and *Calotropis procera.* These plants also include bioactive compounds including hydroquinone and epicatechin, which are used to treat skin pigmentation problems and skin cancer. Numerous kinds of phytochemicals, including alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, polysaccharides, polypeptides, and proteins, have been found in medicinal plant extract, which comprises thousands of secondary metabolites. Over half of all pharmaceuticals marketed for therapeutic use are traditional medicinal plants [10]. Medicinal plants are thought to be crucial for treating a wide range of illnesses. Because of their medicinal qualities and ability to treat both the underlying cause of illness and its symptoms, herbal medicines are frequently referred to as medications. The World Health Organization defined phytomedicines as finished, labeled medical goods that contain an active component,

whether in its raw form or as a plant preparation [11]. Plant species that are utilized worldwide for a variety of reasons, including the treatment of diseases, typically have biological characteristics that are attributed to active chemicals created during secondary metabolism [12].

Many research works conducted recently have alerted the public to the risk and perils posed by pathogenic bacteria that have developed an immunity to antimicrobial agents [13]. More than 100,000 plants have been found worldwide to date, or their potential medical uses have not yet been fully explored or examined. It is anticipated that medicinal plants will become increasingly important, particularly for treating serious illnesses like cancer. Because herbal plants have the potential to be used as medicinal agents both now and in future, it is imperative to verify their efficacy.

The goal of the research project is to use proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and column chromatography to identify and analyze secondary metabolites from *Calotropis procera.* The phytochemical content of *Calotropis procera* leaf powder includes flavonoids, alkaloids, cardenolides, steroids, tannins, glycosides, terpenoids, and saponins [24]. Compounds such as Taraxasterol and β-Amyrin acetate were extracted from *Calotropis procera* bulbs using spectroscopic characterisation and phytochemical screening [25]. Ansari [26] stated that spectral data and chemical reactions have been used to clarify the structures of three oleanane types triterpenes, calotropoleanyl ester, proceroleanenol-A and proceroleanenol-B, that were isolated from the root bark of *Calotropis procera.* These compounds are identified as olean-13(18)-en-3β-yl acetate, olean-13(18)-en-9α-ol, and olean-5,13(18)-diene-3α-ol. Based on spectrum data and chemical reactions, the structures of the new compounds have been discovered as n-triacontan-10β-ol and urs-18α-H-12,20(30)-diene-3β-yl acetate [27]. In addition to the five known compounds—pseudotaraxasterol acetate, taraxasterol, calotropursenyl acetate, stigmasterol, and (E)-octadec-7-enoic acid. Sabrin et al [28] reported the isolation and structural elucidation of four new ursane-type triterpenes named calotroprocerol, calotroproceryl acetate, calotroprocerone, and calotroproceryl acetate. Based on Armin et al [29], the primary constituents of the *C. procera* extract were rutin, catechin, p-coumaric acid, cafeic acid, luteolin, and kaempferol. Using vacuum liquid column chromatography (VLCC) and gravity column chromatography (GCC), the chemicals taraxerol acetate and calotropone, respectively, were found in the ethyl acetate extract of *Calotropis procera* [30]. The completed study will continue

to be an important source for cutting-edge medications for serious illnesses for whom there is no known treatment, and it will play a critical role in the creation of early pharmaceuticals. Sitosterol is one of the isolated substances that aids in lowering the body's cholesterol levels. Finally, by preventing the growth and stimulation of cancer cell, stigmasterol can prevent the formation of many types of malignant cells. Further analysis on biological activities of the isolated compounds is essential.

MATERIALS AND METHODS

Plant collection

The (*Calotropis procera*) roots were collected from a loocation near the Centre for African Medicinal Plants Research North-Eastern University, Gombe Gombe-Bauchi Express way, Lafiyawo Gombe, Nigeria on 10.2881^0 N and 11.0537^0 E and was identified by the Director, Centre for African Medicinal Plants Research Laboratory.

Sample preparation

After being chopped into bits and allowed to air dry for approximately two weeks, the obtained *Calotropis procera* roots were mashed using a porcelain mortar and pestle and then weighed with an electric weighing scale. For the isolation process, a target weight of one kilogram was utilized.

Extraction

Cold maceration for 12 hours was used to extract 10 gram of plant material using 500 mL of hexane and 400 mL of ethyl acetate. The process of macerating *Calotropis procera* using hexane, ethyl acetate, and methanol in order from the non-polar hexane to the polar methanol was prior to the separation of bioactive chemicals from the plant. After a day, the filtrate was collected using a Winchester bottle, a funnel, and fluted Whatman No. 1 filter paper. The extract (filtrate) was concentrated by employing a rotary evaporator to remove the solvent from the filtrate. Then, the concentrate was allowed to air dry and adsorbed onto silica [22].

Column chromatography

Weighing the dehydrated samples was done using a weigh balance. The samples were spread out over 0.9 g of silica gel and allowed to dry until a freely flowing powder was formed. The column

was filled with a 50 g slurry of silica gel that had been made in a 90:10 mL ratio using a mixture of hexane and ethyl acetate. The pre-adsorbed plant extracts were gradiently eluted using ethyl acetate and hexane mixes (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, and 50:50, respectively) after they were added to the column bed. Then, different fractions (CRE-1, CRE -2, CRE -3, CRE -4, CRE -5, CRE -6, CRE -7, CRE -8, CRE -9, CRE -10, CRE -11, CRE -12, CRE -13, CRE -14, and CRE -15 up to CRE -200) were collected and put into vials with the appropriate labels [23].

Thin layer chromatography (TLC)

The dried hexane and ethyl-acetate fractions (desired crystals) were dissolved in a 9:1 hexane to ethyl-acetate mixture in order to reconstitute them. A thin line was carefully drawn across one end of the TLC plates, making sure not to contact the coated surface, in preparation for the experiment. Little spots of the fraction solutions were created using capillary tubes, spaced five millimeters apart (spotter). The vial numbers that contained the proportion utilized were used to assign numbers to the spots. These were (CRE-1, CRE -2, CRE -3, CRE -4, CRE -5, CRE -6, CRE -7, CRE -8, CRE -9, CRE -10, CRE -11, CRE -12, CRE -13, CRE -14, CRE-15, CRE-16, CRE-17, CRE-18, CRE-19, and CRE-20). The plates were then placed such that the selected portions were slightly below the line and transferred with forceps into the developing solutions. The growth setup was covered, and the plates were left to develop for 20 mins. As soon as the solvent stopped rising to the top, the TLC plates were carefully removed with a forceps. After the marking process, the plates were sprayed with a solution that contained a 90%:10% CH_3OH and H_2SO_4 mixture. Using the previously described solution, the plates are sprayed in the charring process. Then, the TLC is heated in a dryer to induce the solvent to flow away from the line and produce a pink and green color [23]. Thin layer chromatographic techniques were used for subsequent purification on similar eluents containing the same components based on their Rf values obtained under the same experimental conditions. Column chromatography purification was performed using various column sizes (2-3 cm diameter), to be packed with silica gel (Merck Art. 5554) in specific solvent systems. After combining those with similar Rf values, 10 fractions with varying Rf values were obtained during the purification process, and they had a milky tint. Additionally, each has a single spot from the thin layer chromatography. Following

spectrum analysis, the ten isolates named CRE-1, CRE-20, CRE-36, CRE-45, CRE-57, CRE-68 and CRE-70, CRE-85, CRE-91, and CRE-110 were examined out of which only CRE-85 and CRE-110 were good compounds [23].

Nuclear magnetic resonance spectroscopy

After they were extracted, 1H NMR spectroscopy was utilized to further identify the structures of the bioactive chemicals in *Calotropis procera* root extract. The NMR samples were made by dissolving the sorted fractions in deuterated chloroform. NMR spectra of the ^{1}H spectra was conducted on a 400 MHz Bruker ADVANCE III NMR spectrometer, and was recorded according to the central line at δ7.26 in the proton NMR spectrum. The spectrum was processed using Bruker NMR academic Topspin software.

RESULTS AND DISCUSSION

Proton NMR characterisation of CRE-110.

Fraction CRE-110 (white crystals) had the following ^{1}HNMR signals: ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.74 (d, J = 2.3 Hz, 1H), 4.61 (s, 1H), 3.19 (dd, J = 11.4, 4.9 Hz, 1H), 2.99 (td, J = 10.8, 4.9 Hz, 1H), 2.35 (t, J = 7.4 Hz, 1H), 2.28 – 2.15 (m, 2H), 2.03 – 1.92 (m, 2H), 1.69 (s, 3H), 1.26 (d, J = 7.4 Hz, 6H), 0.97 (d, J = 5.0 Hz, 7H), 0.94 (s, 3H), 0.82 (s, 3H), 0.75 (s, 3H), 0.68 (d, J = 9.4 Hz, 1H). (Appendix 1; Table 1). A set of terminal, olefinic protons at δH 4.74 and 4.61, an oxymethine proton at δH 3.19, 2.99 and 2.35. group of methylenes at δH 2.28, 2.03 and a set of methyls at δH 1.69 (allylic), 0.94, 0.82, and 0.75. These signals are reminiscent of triterpene proton spectra; specifically, lupane-type triterpenoids.

The HNMR spectroscopic data of the isolated compound were compared with that obtained from literature [[17-19, 23], as presented in Table 1.

Table 1: NMR data of compound CRE-110.

S/N	Shift	δH, J's	[14]	[15]	[16]
1	0.68	d, J = 9.4 Hz, 1H	0.70 (H5)	-	-
2	0.75	s, 3H	0.78 (H24)	0.76 (s, H3-24)	0.74 CH_3
3	0.82	s, 3H	0.82 (H25)	0.82 (s, H3-25)	0.84 CH_3

4	0.94	s, 3H	0.92 (H26)	0.97 (s, H3-23)	0.94 CH_3
5	0.97	d, *J* = 5.0 Hz, 7H	0.96 (H23)	0.97 (s, H3-27)	0.95 CH_3
6	1.26	d, *J* = 7.4 Hz, 6H	0.98 (H27)	1.02 (s, H3-26)	1.01 CH_3
7	1.69	s, 3H	1.71 (H30)	1.68 (s, H3-30)	1.68 (3H, s, H-30)
8	2.03-1.92	m, 2H	-	-	1.53/1.17 (2H, tt, J = 3.0/13.0 Hz, H-22)
9	2.28-2.15	m, 2H	-	-	2.23/1.44 (2H, tt, J = 3.0/12.5 Hz, H-16)
10	2.35	t, *J* = 7.4 Hz, 1H	-	-	2.33 (1H, ddd, J = 2.5/11.5 Hz, H-13)
11	2.99	td, *J* = 10.8, 4.9 Hz, 1H	3.04 (H19)	Two H 3.31 and 3.78 (d, J = 10.7, H28 and H-28')	3.04 (1H, ddd, J = 5.0/11.0 Hz, H-19)
12	3.19	dd, *J* = 11.4, 4.9 Hz, 1H	3.21 (H3)	-	3.10 (1H, t, J = 5.0 Hz, H-3)
13	4.61	s, 1H	4.62 (H29)	H 4.58, (s, H-29 and 29')	4.58 (1H, dd, J = 2.5/2.0 Hz, H-29)
14	4.74	d, *J* = 2.3 Hz, 1H	4.75 (H29)	H 4.68 (s, H-29 and 29')	4.71 (1H, dd, J = 2.5/2.0 Hz, H-29)
15	-	-	-	-	10.5 b. s

The comprehensive literature survey and comparison with different spectral data identified the compound isolated in this research to be betulinic acid.

The ^{1}HNMR spectrum of fraction CRE-110 is presented in Figure 1

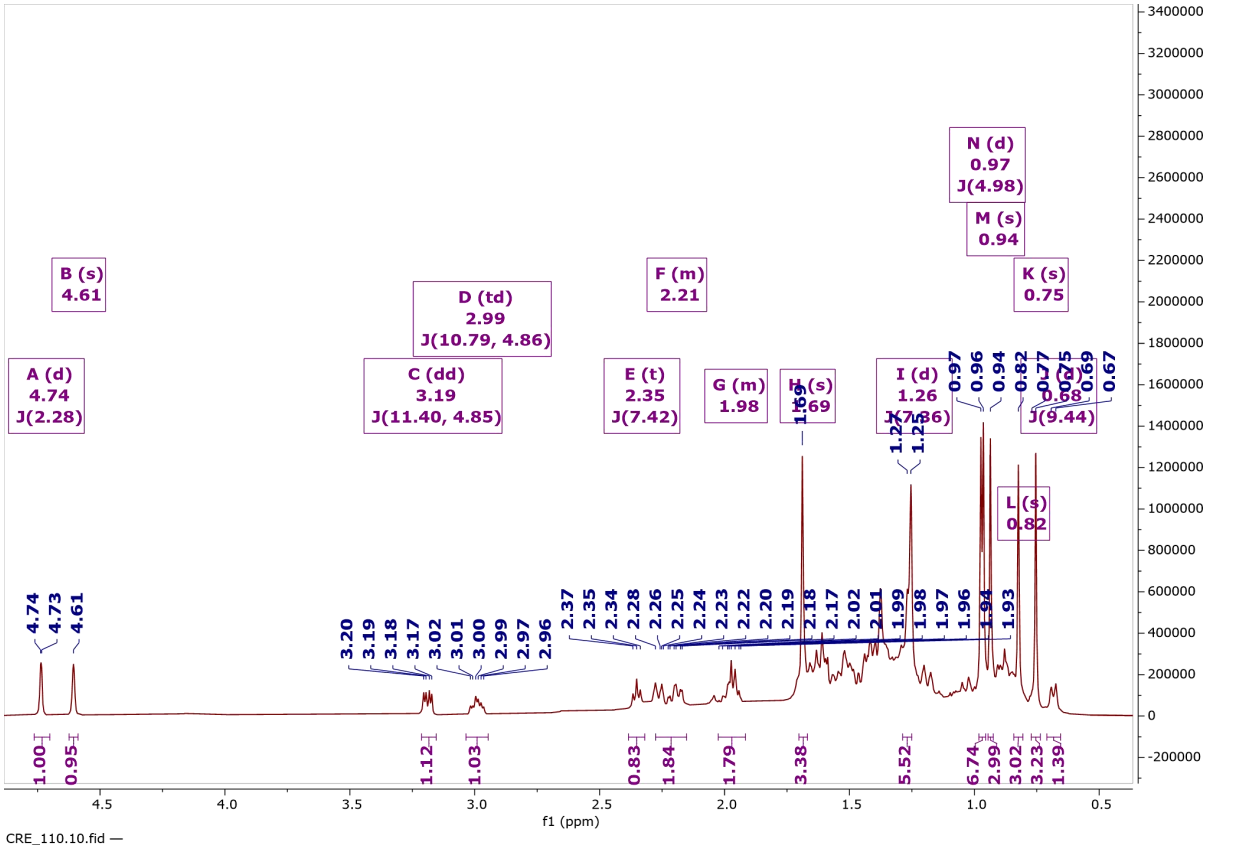

Figure 1. HNMR spectrum of betulinic acid.

The chemical structure of the compound isolated from fraction CRE-110 is presented in Figure 2.

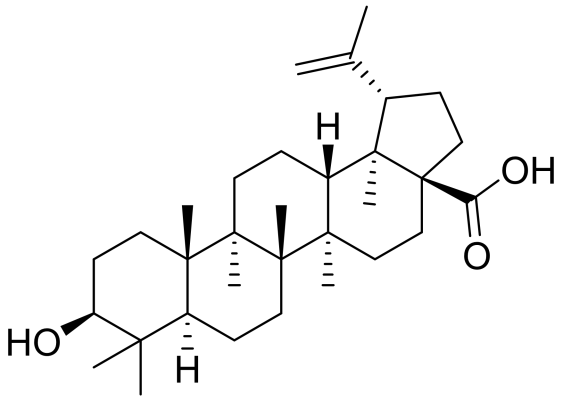

Figure 2. Structure of betulinic acid

HNMR Characterisation of CRE-85 as β-sitosterol and stigmasterol

CRE-85, isolated as fine white crystals, exhibited a positive reaction (purplish colouration) to the Liebermann-Burchard spray, indicating its sterol nature. The NMR data obtained were as follows: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.35 (d, *J* = 5.1 Hz, 1H), 5.15 (dd, *J* = 15.1, 8.6 Hz, 0H), 5.02 (dd, *J* = 15.2, 8.6 Hz, 0H), 3.52 (tt, *J* = 10.6, 4.7 Hz, 1H), 2.38 – 2.17 (m, 4H), 2.00 (ddd, *J* = 21.8, 16.0, 11.2 Hz, 3H), 1.88 – 1.79 (m, *J* = 5.8, 5.3 Hz, 3H), 1.25 (s, 4H), 1.01 (s, 3H), 0.92 (d, *J* = 6.3 Hz, 2H), 0.84 (s, 1H), 0.82 (d, *J* = 4.2 Hz, 1H), 0.80 (s, 2H), 0.69 (d, *J* = 9.1 Hz, 3H). (The spectrum was scaled to the residual deuterated chloroform signal, assumed to resonate at 7.26 ppm). The ^{1}H NMR spectrum of CRE-85 revealed six methyl signals: four methyl peaks at δH 0.69, 1.01, 1.79. one olefinic proton was observed in the ^{1}H NMR spectra of CRE-85 at δH 5.35. Moreover, CRE-85's ^{1}H NMR spectra exhibited a proton corresponding to an oxymethine-type proton as a triplet of doublets at δH 3.52.

The HNMR spectroscopic data of the isolated compound were compared with that obtained from literature [22] as presented in Table 2.

Table 2: NMR data of compound CRE-85: β-sitosterol and Stigmasterol compared against literature reference values.

S/N	Shift	δH, J's	[20]	[21]
1	0.69	d, *J* = 9.1 Hz, 3H	0.661 (dd, 2H of C23)	0.71, 0.73 CH_3
2	0.80	s, 2H	0.800 (dd, 2H of C28)	
3	0.82	d, *J* = 4.2 Hz, 1H	0.811(d, 1H of C25)	
4	0.84	s, 1H	0.827 (d, 1H of C20)	
5	0.92	d, *J* = 6.3 Hz, 2H	-	0.95, CH_3
6	1.01	s, 3H	0.990 (s, 3H of C21).	1.04, CH_3
7	1.25	s, 4H	-	
8	1.88 – 1.79	m, *J* = 5.8, 5.3 Hz, 3H	1.670 (s, 3H of C18)	1.53, 1.86 CH_3
9	2.00	ddd, *J* = 21.8, 16.0,	-	

		11.2 Hz, 3H		
10	2.38 – 2.17	m, 4H	-	
11	3.52	tt, *J* = 10.6, 4.7 Hz, 1H	3.506 (m, 1H of C3)	3.54 CH
12	5.02	dd, *J* = 15.2, 8.6 Hz, 0H	-	5.04 CH
13	5.15	dd, *J* = 15.1, 8.6 Hz, 0H	-	5.18 =CH
14	5.35	d, *J* = 5.1 Hz, 1H	5.337 (t,1H of C6)	5.38 =CH

The comprehensive literature survey and comparison with different spectral data identified the compound in CRE-85 isolated in this research to be β-sitosterol and stigmasterol.

The HNMR spectrum of fraction CRE-85 is presented in Figure 3

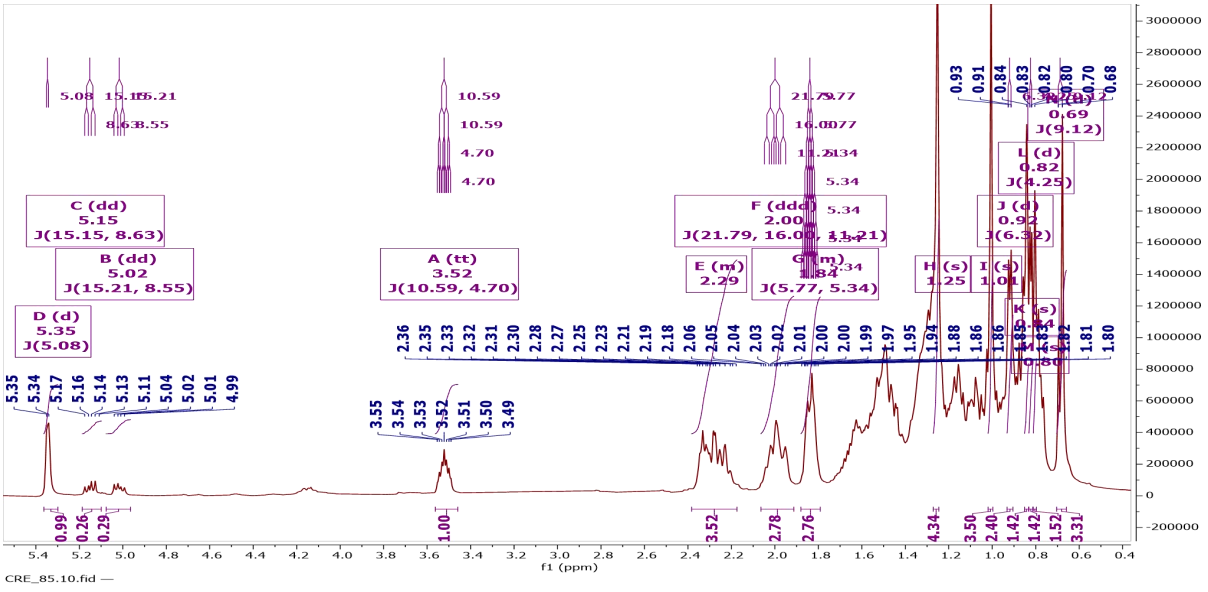

Figure 3:. HNMR spectrum of stigmasterol and β-sitosterol

The chemical structures of the compound isolated from fraction CRE-85 which are stigmasterol and β-sitosterol are presented in Figure 4.

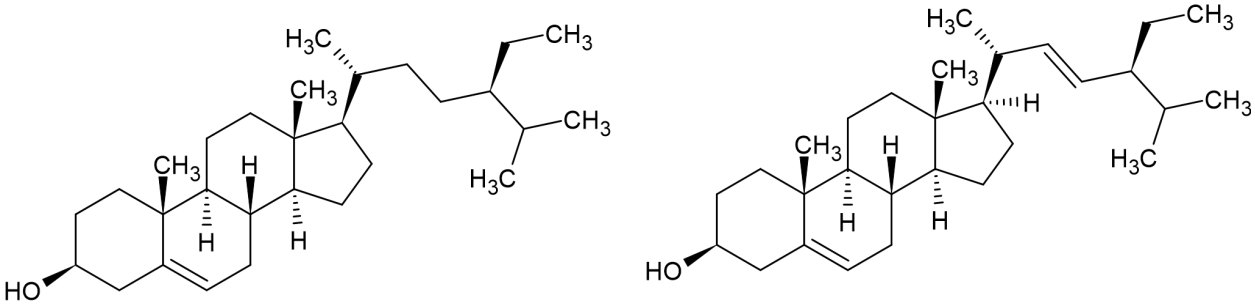

Figure 4: Structure of stigmasterol and β-sitosterol

CONCLUSION

Betulinic acid and a combination of β-Sitosterol and Stigmasterol were among the compounds that were extracted from *Calotropis procera.* Physical and spectroscopic approaches were used for the identification of the structure. By contrasting the physical characteristics with those reported in literature, the characterisation was made possible. There should be more research done on the pharmacological actions.

Recommendation

Following the successful extraction and characterization of bioactive compounds from *Calotropis procera,* it is recommended that more Calotropis species be studied and the extracted chemicals be commercialised. This will boost the Nigerian economy. Additionally, the compound's biological activity needs to be looked at.

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